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Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an *in vivo* murine model of cardiac hypertrophy

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ABSTRACT **To study the mechanisms that activate expression of the atrial natriuretic factor (ANF) gene during pressure-induced hypertrophy, we have developed and characterized an *in vivo* murine model of myocardial cell hypertrophy. We employed microsurgical techniques to produce a stable 35- to 45-mmHg pressure gradient across the thoracic aorta of the mouse that is associated with rapid and transient expression of an immediate-early gene program (c-*fos*/c-*jun*/*junB*/Egr-1/nur-77), an increase in heart weight/body weight ratio, and up-regulation of the endogenous ANF gene. These responses that are identical to those in cultured cell and other *in vivo* models of hypertrophy. To determine whether tissue-specific and inducible expression of the ANF gene can be segregated, we used a transgenic mouse line in which 500 base pairs of the human ANF promoter region directs atrial-specific expression of the simian virus 40 large tumor antigen (T antigen), with no detectable expression in the ventricles. Thoracic aortic banding of these mice led to a 20-fold increase in the endogenous ANF mRNA in the ventricle but no detectable expression of the T-antigen marker gene. This result provides evidence that atrial-specific and inducible expression of the ANF gene can be segregated, suggesting that a distinct set of regulatory cis sequences may mediate the up-regulation of the ANF gene during *in vivo* pressure overload hypertrophy. This murine model demonstrates the utility of microsurgical techniques to study *in vivo* cardiac physiology in transgenic mice and should allow the application of genetic approaches to identify the mechanisms that activate ventricular expression of the ANF gene during *in vivo* hypertrophy.**

In response to diverse stimuli, such as hypertension, valvular heart disease, and endocrine disorders, the myocardium adapts to increased workloads through the hypertrophy of individual muscle cells (for a review, see refs. 1 and 2). Although the signaling mechanisms that mediate the hypertrophic response of cardiac muscle cells remain unclear, transcriptional activation of cardiac target genes, including contractile proteins and embryonic markers, appears to play a pivotal role in this adaptive response (3, 4). In this regard, the reactivation of atrial natriuretic factor (ANF) gene expression in ventricular cells occurs in response to diverse hypertrophic stimuli (genetic, hormonal, volume overload, pressure overload, hypertension, etc.) in multiple species (5–11), including humans, and could be considered one of the conserved features of ventricular cell hypertrophy.

To study the transcriptional regulation of cardiac genes, workers in our laboratory (3, 12, 13) and others (14–16) have extensively characterized cultured myocardial cell models in which several features of hypertrophy can be induced after stimulation with defined agents, such as α-adrenergic agonists (3, 12, 14–17) or endothelin 1 (13). In this model, the inducibility of a constitutively expressed contractile protein gene, myosin light chain 2 (MLC-2), is mediated by conserved cis regulatory elements (HF-1 and HF-2), which also are involved in cardiac-specific expression (17). Extensive mutagenesis studies have been unable to segregate cardiac-specific and inducible expression within a 250-base-pair (bp) fragment of the MLC-2 promoter, which confers both responses (17, 18). Whether tissue-specific regulatory mechanisms have a similar role in inducible expression of an embryonic marker, such as the ANF gene, is a question of considerable interest. Since previous studies have utilized neonatal rat myocardial cell models of hypertrophy to study cardiac gene expression (3, 4, 12–17), it would also be of particular value to critically examine the relevance of studies in *in vitro* models to pressure overload hypertrophy in the *in vivo* setting. The current study describes the development of an *in vivo* murine model of pressure overload hypertrophy to directly address this question in normal and transgenic mice.

METHODS

Surgical Procedures. Eight-week-old adult mice (C57/BL6 × SJL; The Jackson Laboratory), weighing 18–22 g, were anesthetized with a mixture of ketamine (100 mg/kg; intraperitoneal injection), xylazine (5 mg/kg; intraperitoneal injection), and morphine (2.5 mg/kg; intraperitoneal injection). Under a dissecting microscope (model ZDX-80; Scope Instruments, San Diego), animals were placed in the supine position and a midline cervical incision was made to expose the trachea and carotid arteries by microsurgical techniques. After successful endotracheal intubation, the cannula was connected to a volume cycled rodent ventilator (Harvard Apparatus) on supplemental oxygen with a tidal volume of 0.2 ml and respiratory rate of 110 per min. Both right and left carotid arteries were cannulated with flame stretched PE 50 tubing. Catheters were connected to modified P50 Statham transducers, and the transducer dome was modified to accept a 23-gauge needle bore at the spout, which was connected to the catheter. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision, and aortic constriction was performed by tying a 7-0 nylon suture ligature against a 27-gauge needle to yield

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Abbreviations: ANF, atrial natriuretic factor; MLC-2, myosin light chain 2; TAC, transverse aortic constriction; T antigen, large tumor antigen; SV40, simian virus 40.

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a narrowing 0.4 mm in diameter when the needle was removed and a reproducible transverse aortic constriction (TAC) of 65–70%. For chronic studies, neither carotid artery was cannulated, the pneumothorax was evacuated, and the animals were extubated and allowed to recover. The overall mortality rate for animals that recovered from anesthesia in this chronic thoracic model was 10%, while the operative mortality for both the acute and chronic studies was 4%.

Northern and Immunoblotting Analyses. Immunoblotting, immunofluorescence, and Northern blotting analyses were performed by modifications of described methods (3, 12, 13).

RESULTS AND DISCUSSION

The present study reports the development of a microsurgical technique for the reproducible induction of pressure overload hypertrophy with an acceptable mortality (10%), and a success rate (>70%) that compares favorably with larger animal models (19, 20). In the course of operating on >300 mice, a number of critical experimental variables were optimized (intubation and respiratory control, the duration and extent of anesthesia, intraoperative technique, and catheter design/placement). A schematic of the surgical preparation is displayed in Fig. 1 (*Upper Left*). To determine the diameter of the major vessels, a silastic elastomer was infused under pressure (100 mmHg; 1 mmHg = 133 Pa) to form a cast of the arterial tree in one adult mouse of the same approximate weight as in these studies (*Inset*). The diameters of the right and left carotid arteries at the site of cannulation were 0.56 and 0.48 mm, respectively, while the diameter of the transverse aorta at the site of where constrictions were performed was 1.2 mm.

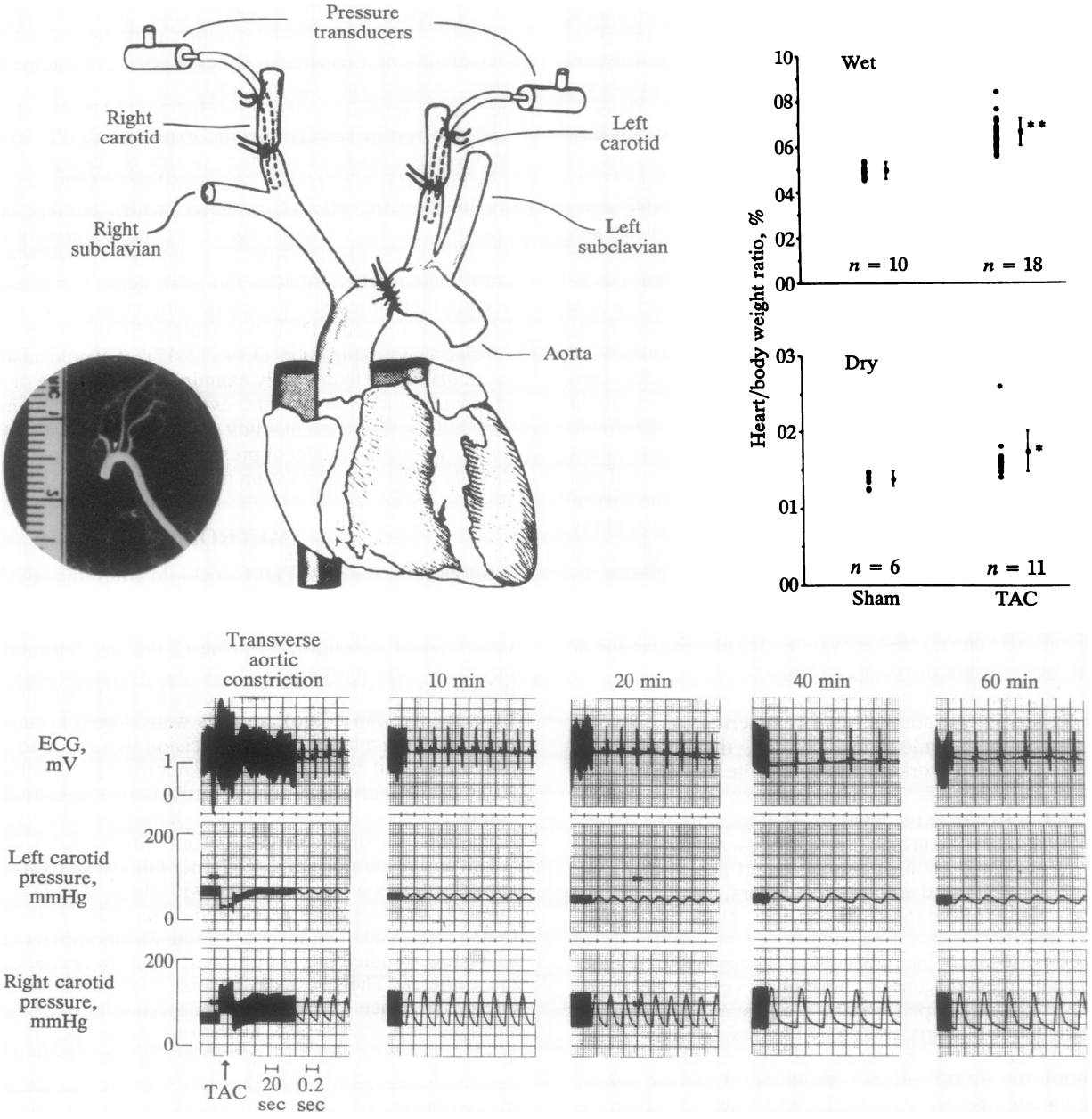

FIG. 1. Induction of *in vivo* pressure overload in murine myocardium after transverse aortic constriction. (*Upper Left*) Schematic of the surgical preparation. (*Inset*) Silastomer cast of the mouse aortic tree. (*Lower*) Electrocardiographic (ECG) and hemodynamic effects before and after TAC. (*Upper Right*) Effects of chronic TAC on heart weight/body weight ratio. For the assessment of heart weight, the mice were divided into two groups—sham operated and TAC. After 7 days of TAC, a 33% increase in wet weight and a 24% increase in dry heart weight/body weight ratio were observed. Data are expressed as individual experiments and mean ± SD. *, $P < 0.05$; **, $P < 0.001$ TAC vs. sham.

Hemodynamic Response to TAC. The hemodynamic effects of acute constriction were followed by monitoring the pressure difference between the two carotid arteries. Original pressure tracings of simultaneous electrocardiograms, left and right carotid pressures before and after TAC are shown (Fig. 1 *Lower*). Before aortic constriction, systolic pressure in both carotids was 75 mmHg. During the period of total occlusion, the distal pressure fell while the peak proximal aortic pressure (equal to left ventricular systolic pressure) was 145 mmHg. After removal of the 27-gauge needle, the left carotid (distal) systolic pressure was shown to be 70 mmHg, while the right carotid (proximal) systolic pressure was 115 mmHg, resulting in a 45 mmHg gradient (Fig. 1). Despite fluctuations in the aortic pressure, the gradient across the stenosis was maintained, and the rhythm remained constant throughout the subsequent 60-min period.

Average hemodynamic data (mean ± SD) from nine experiments are summarized below. At baseline, prior to TAC, proximal systolic and mean aortic pressure were 64 ± 13 and 47 ± 13 mmHg, respectively. During placement of the 7-0 suture ligature, with the transverse aorta occluded, peak systolic pressure reached 136 ± 11 mmHg, which promptly decreased after removal of the 27-gauge needle. Proximal pressure rapidly stabilized and at 15 min was 101 ± 20 mmHg, resulting in an average systolic pressure difference before and after constriction of 37 ± 15, with a peak-to-peak systolic gradient of 43 ± 13 mmHg. In these experiments, the maximal systolic pressure during occlusion of the transverse aorta was 155 mmHg. In contrast to ascending aortic constriction, this experimental model of constriction avoids excessive overload on the left ventricle because of the position of the innominate artery proximal to the constriction, allowing for a low-resistance outlet.

Physiologic and Morphologic Assessment of Hypertrophy After Chronic Aortic Constriction. After 7 days of chronic pressure overload, a 41% increase in heart wet weight (n = 18, TAC; n = 10, sham) and a 30% increase in heart dry weight (n = 11, TAC; n = 6, sham) occurred in the hypertrophied hearts compared to sham-operated hearts ($P < 0.001$ and $P < 0.05$, respectively). A 33% increase in wet and a 24% increase in dry heart weight/body weight ratio resulted from chronic constriction of the thoracic aorta (Fig. 1 *Upper Right*). The cross-sectional area of myocytes (mean cell area at the nucleus) increased from 121.9 ± 43.1 to 163.7 ± 56.9 ($P < 0.001$) μm^2 in the hypertrophied ventricles (n = 4 or 5). No evidence for ischemic necrosis or fibrous scar formation was observed in any region (inner, mid, outer wall) of the left ventricle or papillary muscles.

Induction of Immediate-Early Gene Program. *In vivo* pressure overload rapidly activated a program of immediate-early gene expression (Fig. 2), including c-*fos*/c-*jun*/*jun-B* (21)/Egr-1 (12)/nur77 (22), which is an essential feature of both *in vivo* and *in vitro* models of hypertrophy (12, 23–26). A *fos*-related antigen, *fra-1*, which is inducible during serum stimulation of several cell types and is usually coregulated with the other immediate-early genes (27), was not expressed after the induction of pressure overload. Thus, a distinct subset of immediate-early genes was rapidly induced after *in vivo* pressure overload in murine myocardium. This pattern appeared to be identical to that observed during α-adrenergic-mediated hypertrophy of neonatal rat myocardial cells, and distinct for cardiac cells, as serum stimulation of fibroblasts induces a different pattern of immediate-early gene expression, which includes the activation of *fra-1* (27).

The identification of similar patterns of immediate-early gene expression during *in vivo* pressure overload hypertrophy of adult myocardium and agonist-mediated hypertrophy of neonatal rat myocardial cells suggests the possibility that conserved mechanisms might orchestrate cardiac gene expression, independent of the hypertrophic stimulus. Since

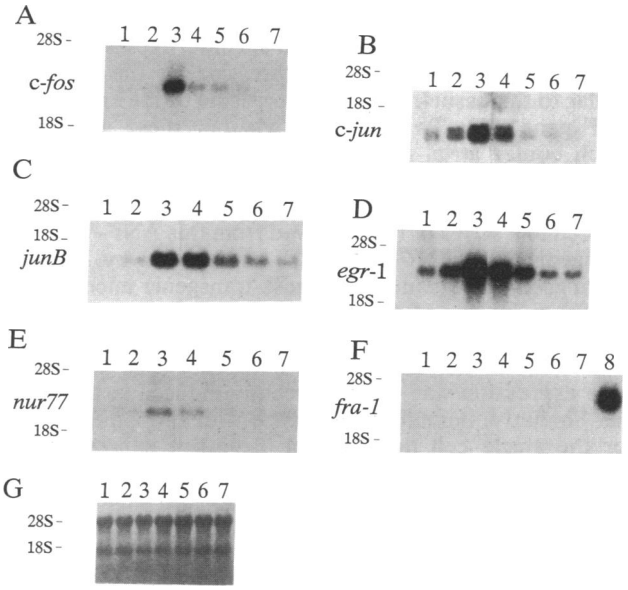

FIG. 2. Induction of an immediate-early gene program *in vivo* in murine myocardium during pressure overload. Results are representative of n = 3 or greater for each time point. (*A*) c-*fos*. (*B*) c-*jun*. (*C*) *junB*. (*D*) Egr-1. (*E*) nur77. (*F*) *fra-1*. (*G*) Methylene blue staining of the nylon filters after transfer. Lanes: 1, sham (time 0); 2, sham (60 min); 3, 15 min TAC; 4, 60 min TAC; 5, 2 hr TAC; 6, 4 hr TAC; 7, 6 hr TAC. RNA isolated from serum-stimulated NIH 3T3 cells (lane 8) served as a positive control for the detection of *fra-1*.

the induction of constitutively expressed contractile protein genes (e.g., the MLC-2 gene) and the activation of a program of embryonic gene expression (e.g., the ANF gene) occur during many forms of *in vitro* and *in vivo* hypertrophy, the possibility exists that conserved mechanisms might mediate the inducibility of these central genetic features of myocardial cell hypertrophy. However, it should be emphasized that the precise role of α-adrenergic stimulation and immediate-early genes in the activation of the *in vivo* hypertrophic response is currently unknown.

Induction of the Endogenous ANF Gene. Thoracic aortic banding of the mouse aorta leads to a marked increase in the expression of the ANF gene in the hypertrophied ventricle, consistent with the activation of an embryonic program of gene expression (Fig. 3). After 1 week of thoracic banding, ANF mRNA levels are significantly increased to >20-fold of the control level, similar to results obtained in other *in vivo* and *in vitro* models of myocardial cell hypertrophy (5–11). This result provided further evidence for the activation of the hypertrophic response in the mouse model and suggested its utility in the study of mechanisms that mediate transcriptional activation of the ANF gene during chronic pressure overload.

Studies in ANF–Large Tumor Antigen (T antigen) Transgenic Mice. For these studies, we used a well characterized line of transgenic mice that harbor an ANF–T-antigen fusion gene (28, 29). The transgene consists of a 500-bp fragment of the human ANF 5′ flanking region fused to coding sequences

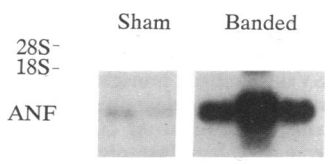

FIG. 3. Induction of ANF gene expression in ventricular myocardium after 7 days of thoracic aortic banding. The levels of ANF mRNA were examined by Northern blotting of total RNA (10 μg) with a rat ANF cDNA probe.

for the simian virus 40 (SV40) T antigen. The 500-bp ANF promoter fragment directs atrial-specific expression of T antigen, with no detectable expression in the ventricle, similar to the tissue-restricted expression of the endogenous ANF gene (30). To determine whether these sequences, which confer atrial-specific expression of the ANF gene, would also be sufficient to confer inducible expression during ventricular cell hypertrophy, thoracic banding (7 days) was induced in a series of mice derived from this ANF–T-antigen transgenic line (ANF–TAG 52). As assessed by Northern blotting, thoracic banding of these transgenic mice led to a marked induction of the endogenous ANF gene (Fig. 4*A*). However, as assessed by either Northern blotting (Fig. 4*B*) or immunoblotting with T-antigen antiserum (Fig. 4*C*), transgene expression was not significantly induced in the ventricle. Similarly, immunofluorescence with antibodies suitable for the single cell detection of T-antigen expression (33) resulted in no evidence of positive staining in the ventricles, while the atria showed markedly positive staining for T antigen (data not shown). Taken together, these results suggest that two important functions of the ANF promoter region—i.e., the maintenance of atrial-restricted expression and the up-regulation of ANF gene expression during ventricular hypertrophy—can be segregated during mechanical overload in this transgenic mouse line. Since previous studies of the rat cardiac MLC-2 gene have suggested that a similar set of conserved cis regulatory elements can confer both cardiac-specific and inducible expression during myocardial cell hypertrophy (17), the results of the present study suggest that divergent mechanisms may mediate the induction of an embryonic gene, such as the ANF gene, and the up-regulation of a constitutively expressed contractile protein—i.e., MLC-2.

It remains to be determined whether a similar set of cis regulatory sequences will mediate the activation of ANF gene expression in the embryonic ventricle and the induction of the ANF gene in the hypertrophied adult ventricular cell *in vivo*. Previous studies from our laboratory have documented that T antigen can be expressed to relatively high levels in neonatal rat ventricular cells without the loss of cell viability (33). Thus, it would appear that the T-antigen mRNA is not unstable in the ventricular cells and that this does not represent a significant limitation to the current studies. In addition, mice harboring a myosin heavy chain promoter–T-antigen construct have been demonstrated to express T antigen in the ventricle in a tissue-specific fashion (L.F., unpublished observations). This also would argue that the expression of the T antigen in the transgenic mice is not lethal and/or toxic. Finally, it is possible, although unlikely, that the observed effects are simply due to the site of integration. The frequency of ANF–T-antigen founders in the initial generation of the mice was not consistent with atrial expression of the T antigen being a rare, selected event. In addition, the position-dependent effect would have to selectively affect expression in the atrium versus the ventricle, since there is a high level of expression of T antigen in the atrium, as noted in our Northern blotting studies with total RNA.

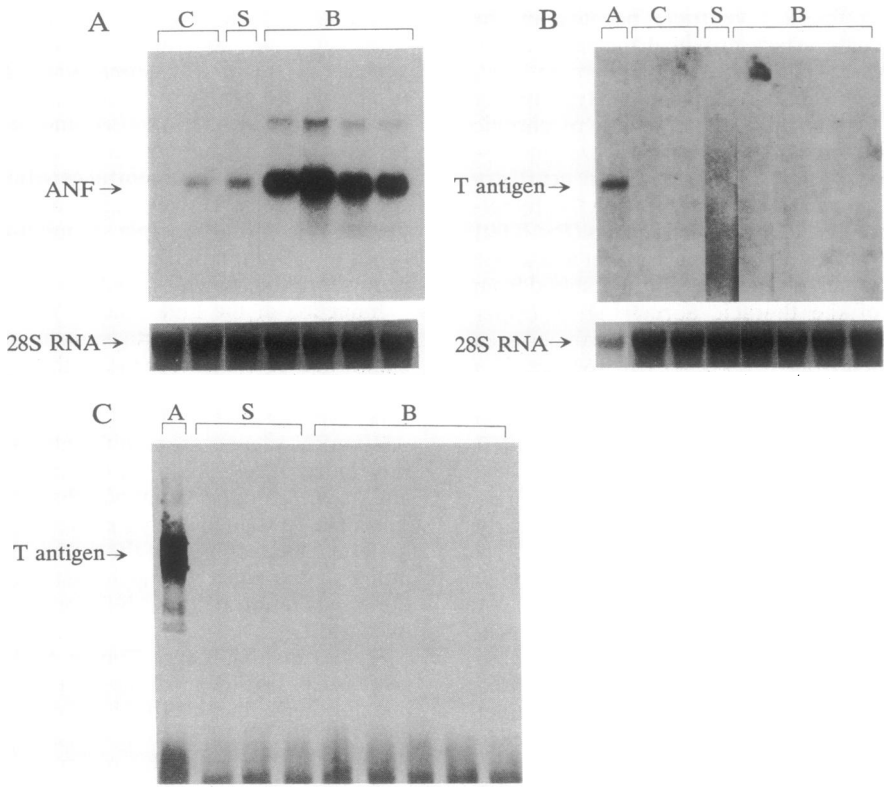

FIG. 4. A 500-bp fragment of the human ANF promoter confers atrial-specific but not inducible ventricular expression of a SV40 T-antigen marker gene in transgenic mice. Thoracic aortic banding was performed in a well-characterized line of transgenic mice (ANF–T antigen) that harbor an ANF–SV40 T-antigen transgene consisting of 500 bp of the human ANF promoter fragment fused to the protein coding sequences of the SV40 T-antigen gene. Previous studies have documented the atrial-specific expression of the T-antigen marker gene. Northern blots were prepared as described. The following cDNA probes were used: rat ANF; 700-bp *Hin*dIII, *Bam*HI digest of pGEMANF (generous gift of C. Glembotski, Molecular Biology Institute, San Diego State University). T antigen: 526-bp *Hin*dIII fragment of pKSV10 (Pharmacia). (*A* and *B*) Northern blot analysis of the expression of the endogenous ANF gene (*A*) and SV40 T-antigen marker gene (*B*) in nonoperated (lanes C), sham-operated (lanes S), and thoracic-banded transgenic mice (7 days) (lanes B), with atrial RNA as a positive control for the T-antigen probe (lane A). Methylene blue staining of nylon filter is shown below each lane. (*C*) Immunoblotting analyses of T-antigen expression in ANF–T-antigen transgenic mice after 7 days of thoracic banding (31). Immunoblotting of T antigen was visualized with a horseradish peroxidase–diaminobenzidine coupled reaction, as described (32).

Since the expression of T antigen in the intact embryonic ventricle may be a lethal phenotype, the currently available ANF–T-antigen lines may not be optimal to directly address this question. Although none of the established ANF–T-antigen lines expresses T antigen in the embryonic ventricle (28), a few transgenic mice have been observed to express T antigen in the neonatal ventricle, which is associated with lethality in the newborn (29).

Previous studies in cultured myocardial cells have documented that a 638-bp fragment of the rat ANF promoter is sufficient to confer inducible expression to a luciferase reporter gene (31). Since the human and rat ANF promoters are highly conserved (>95% within this region), one would anticipate that the results with the rat ANF promoter may be analogous to the human ANF promoter fragment in the transient assays in cultured myocardial cell models of hypertrophy. The inability of the 500-bp human ANF promoter fragment to confer inducible expression in a bona fide *in vivo* model of hypertrophy would suggest that the sequences that are important for inducible expression may lie between −638 and −500 of the human and rat ANF 5′ flanking region. Subsequent truncations of the rat ANF promoter to −323 reveal that inducible expression is lost, implicating a 315-bp fragment as the critical region in the transition between atrial-specific and inducible expression, consistent with the findings in the present study.

SUMMARY

In summary, the development of a reproducible, low mortality *in vivo* model of pressure overload hypertrophy in murine myocardium should ultimately afford the opportunity to directly assess the role of specific signaling molecules in the acquisition of defined features of the hypertrophic response. Transgenic models have been valuable in studying the role of specific genes in the growth and development of other tissues (for review, see ref. 34) and, more recently, in the heart (35, 36). The development of microsurgical techniques to induce bona fide *in vivo* pressure overload hypertrophy in mice and to monitor the hemodynamic effect should now allow a critical evaluation of the signaling mechanisms that transduce a mechanical stimulus into the biochemical signals that activate cardiac gene expression. In addition, it should lead to assessment of the role of specific cis elements and trans-acting factors that activate expression of the ANF gene and other important cardiac target genes during the hypertrophic process *in vivo*. Finally, these studies demonstrate the feasibility of using microsurgical techniques for *in vivo* studies of cardiac physiology in transgenic mice, which should allow the further development of genetic approaches to study other complex problems in the cardiovascular system.

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